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(54) Title: ASSAYS AND PEPTIDE SUBSTRATE FOR DETERMINING AGGREGAN DEGRADING METALLO PROTEASE ACTIVITY (57) Abstract This invention is directed to assays to determine the presence or absence of proteins that exhibit aggrecanase or ADMP activity. This invention also relates to peptides that act as a substrate for ADMPs, their use in various assays to determine the presence or absence of ADMP activity, and their use as inhibitors of ADMP activity.		

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TITLE**ASSAYS AND PEPTIDE SUBSTRATE FOR DETERMINING AGGREGAN
DEGRADING METALLO PROTEASE ACTIVITY**

10

FIELD OF THE INVENTION

This invention is directed to various assays for determining aggrecanase or aggrecan degrading metallo protease (ADMP) activity. This invention also relates to a peptide that acts as a substrate for ADMPs, its use in
15 various assays to determine the presence or absence of (ADMP) activity, and its use as an inhibitor of ADMP activity.

BACKGROUND OF THE INVENTION

20

Aggrecan is the major proteoglycan of cartilage and provides this tissue with its mechanical properties of compressibility and elasticity. In arthritic conditions one of the earliest changes observed in cartilage morphology is the depletion of aggrecan [Mankin et al. (1970) J. Bone
25 Joint Surg. 52A, 424-434], which appears to be due to an increased rate of degradation.

The aggrecan molecule is composed of two N-terminal globular domains, G1 and G2, which are separated by an approximately 150 residue interglobular domain (IGD),
30 followed by a long central glycosaminoglycan (GAG) attachment region and a C-terminal globular domain, G3 [Hardingham et al. (1992) in Articular Cartilage and Osteoarthritis: Aggrecan, The Chondroitin Sulfate/Keratan Sulfate Proteoglycan from Cartilage (Kuettner et al.) pp. 5-
35 20, Raven Press, New York and Paulson et al. (1987) Biochem. J. 245, 763-772]. These aggrecan molecules interact through the G1 domain with hyaluronic acid and a link protein to form large molecular weight aggregates which are trapped within the cartilage matrix [Hardingham et al. (1972)

- 5 Biochim. Biophys. Acta 279, 401-405, Heinegard et al.
(1974) J. Biol. Chem. 249, 4250-4256, and Hardingham, T.E.
(1979) Biochem. J. 177, 237-247]. Loss of aggrecan from
cartilage in arthritic conditions involves proteolytic
cleavage of the aggrecan core protein within the IGD,
10 producing a N-terminal G-1 fragment that remains bound to
hyaluronic acid and the link protein within the matrix,
releasing a large C-terminal GAG-containing aggrecan
fragment that diffuses out of the cartilage matrix. Loss of
the C-terminal fragment results in cartilage deficient in
15 its mechanical properties. This deficiency arises because
the GAGs which are present on the C-terminal portion of the
aggrecan core protein are the components of aggrecan that
impart the mechanical properties to the molecule through
their high negative charge and water binding capacity.
- 20 Two major sites of proteolytic cleavage have been
identified within the IGD, one between amino acid residues
Asn³⁴¹-Phe³⁴² and the other between amino acid residues
Glu³⁷³-Ala³⁷⁴ (human sequence enumeration). Although G1
fragments formed by cleavage at the Asn³⁴¹-Phe³⁴² site and at
25 the Glu³⁷³-Ala³⁷⁴ site have been identified within articular
cartilage [Flannery et al. (1992) J. Biol. Chem. 267, 1008-
1014], the only N-terminus identified on GAG-containing
aggrecan C-terminal aggrecan fragments in synovial fluids of
patients with osteoarthritis [Sandy et al. (1992) J. Clin.
30 Invest. 69, 1512-1516], inflammatory joint disease
[Lohmander et al. (1993) Arthritis Rheum. 36, 1214-1222] and
in the media from cartilage explant and chondrocyte cultures
stimulated with interleukin-1 or retinoic acid [Sandy et al.
(1991) J. Biol. Chem. 266, 8198., Sandy et al. (1991) J.
35 Biol. Chem. 266, 8683-8685., Leulakis et al. (1992) Biochem.
J. 264, 589-593., Ilic et al. (1992) Arch. Biochem.
Biophys. 294, 115-122., Lark et al. (1995) J. Biol. Chem.
270, 2550-2556.] is ARGSVIL, indicating that they were
formed by cleavage between amino acid residues Glu³⁷³-Ala³⁷⁴.

5 These observations suggest that cleavage at this site may be responsible for cartilage degradation.

Although many matrix metalloproteases (MMP-1, -2, -3, -7, -8, -9 and 13) have been shown to cleave in vitro at the Asn³⁴¹-Phe³⁴² site, digestion of aggrecan with a
10 number of these purified proteases has not resulted in cleavage at the Glu³⁷³-Ala³⁷⁴ site [Fosang et al. (1992) J. Biol. Chem. 267, 19470-19474., Flannery et al. (1992) J. Biol. Chem. 267, 1008-1014., Fosang et al. (1993) Biochem. J. 295, 273-276., Fosang et al. (1996) FEBS
15 Lett. 380, 17-20., Flannery et al. (1993) Orthop. Trans. 17, 677., and Fosang et al. (1994) Biochem. J. 305, 347-351]. Therefore, cleavage at this site has been attributed to a novel, proteolytic activity, "aggrecanase".

20 In addition to the Glu³⁷³-Ala³⁷⁴ bond within the interglobular domain of aggrecan, four potential aggrecanase-sensitive sites have been identified within the C-terminus of the aggrecan core protein [Loulakis et al. (1992) Biochem. J. 264, 589-593. and Sandy et al.
25 (1995) Acta Orhtop Scand (Suppl 266) 66, 26-32]. Although cleavage at these sites which are not within the interglobular domain would not be expected to release the major portion of the aggrecan molecule from the matrix, they may be involved in earlier processing of aggrecan
30 within the matrix.

It follows from the foregoing considerations that a sensitive and specific assay that can detect such aggrecanase activity of aggrecan degrading metallo
proteases (ADMPs) would be beneficial in helping to
35 identify inhibitors of members of the ADMP family, which could serve as potential therapeutic agents for treating aggrecanase-related disorders cited above.

5

SUMMARY OF THE INVENTION

A preferred embodiment of the invention provides assays that determine the presence of aggrecan degrading metallo protease (ADMP) activity.

10 A preferred embodiment of the invention provides an assay using purified native aggrecan or recombinant aggrecan as the substrate and monitoring product generation via a direct enzyme-linked immunosorbent assay (ELISA) using neoepitope antibodies to detect the new N-
15 terminus or new C-terminus on aggrecan fragments formed by specific cleavage at an ADMP-sensitive site in the aggrecan core protein.

A preferred embodiment of the invention provides peptides that have been found to act as substrates for
20 the family of aggrecan degrading metallo proteases (ADMPs). One peptide, based on the human aggrecan sequence around the Ala373-Glu374 ADMP-sensitive site has the sequence:

QTVTWPDMELPLPRNITEGE-ARGSVILTVKPIFEVSPSPL

25 (SEQ ID No:1)

A second peptide, based on the bovine aggrecan sequence around the Ala373-Glu374 ADMP-sensitive site has the sequence:

QTVTWPDVLELPLPRNITEGE-ARGSVILTAKPDFEVSPTAPE

30 (SEQ ID NO:2)

Both peptides are capable of being cleaved at this specific recognition site by members of the family of ADMP proteins. A third peptide, based on the human aggrecan sequence around the Ala1714-Gly1715 ADMP-
35 sensitive site has the sequence:

ITFVDTSLEVTPTTFKEEE-GLGSVELSGLPSGELGVSGT

(SEQ ID NO:3)

and is capable of being cleaved at this specific recognition site by the family of ADMP proteins.

5 A preferred embodiment of the invention provides assay formats and methods of utilizing these peptide substrates for the detection and quantification of ADMP activity.

10 A preferred embodiment of the invention provides a modified version of the peptide substrates and a method for their use as an inhibitor of ADMP activity.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Preferred embodiments of the invention have been chosen for purposes of illustration and description, but are not intended in any way to restrict the scope of the invention. The preferred embodiments of certain aspects of the invention are shown in the accompanying drawings
20 described below.

Figure 1. Shows the activity of a biotinylated form of the 41-mer peptide substrate (41-PS) against ADMP enzymatic activity using the microplate assay format and
25 the inhibition of that activity by a hydroxymate inhibitor compound.

Figure 2. Shows the activity of a biotinylated form of the 41-mer peptide substrate (41-PS) against ADMP
30 enzymatic activity using the HPLC assay format.

Figure 3. Shows the activity of the biotinylated form of the 41-mer peptide substrate (41-PS) against ADMP enzymatic activity using the microplate assay format and
35 the inhibition of that activity by the 30-mer inhibitor peptide (30-IP), QVTWPDMEPLPRNITEGQARGSVILTVK-Biotin, the sequence of which is based upon the sequence of the 41-PS.

5 DETAILED DESCRIPTION OF THE INVENTION

A family of aggrecan degrading metallo protease (ADMP) proteins cleave the aggrecan core protein at the Glu³⁷³-Ala³⁷⁴ peptide bond and thus exhibit the enzymatic activity referred to as "aggrecanase" activity [Flannery
10 et al. (1992) J. Biol. Chem. 267, 1008-1014]. The presence of ADMP enzymatic activity can be determined by monitoring the production of aggrecan fragments generated exclusively by cleavage at the Glu³⁷³-Ala³⁷⁴ peptide bond within the aggrecan
15 fragments are detected by using neoepitope antibodies to the new N-terminus or new C-terminus on fragments produced by specific cleavage at this ADMP-sensitive site. The neoepitope antibodies used encompass but are not limited to, the BC-3 monoclonal antibody (Hughes,
20 C.E., et al., Biochem. J. 306:799-804, 1995) as first described in U.S. Provisional Patent Application Serial Number 60/006,684 and subsequently described in U.S. Patent Application Serial Number 08/743,439.

ADMP activity may also be detected by monitoring the
25 production of fragments formed by cleavage at alternative ADMP-sensitive sites using neoepitope antibodies to the new C-terminus or to the new N-terminus generated by ADMP-specific cleavage at these sites. Alternative sites in the aggrecan core protein encompass, but are not
30 limited to, the E1545-G1546, E1714-G1715, E1819-A1820, or E1919-L1920 bond (numbering based on the human aggrecan core protein sequence).

A preferred assay format involves using purified native aggrecan or recombinant aggrecan as the substrate
35 with product detection via a direct enzyme-linked immunosorbent assay (ELISA), herein referred to as the "Problot assay", using neoepitope antibodies to the new C-terminus or new N-terminus on aggrecan fragments generated upon specific cleavage at ADMP-sensitive sites

5 within the aggrecan core protein. Alternative sites in
the aggrecan core protein encompass, but are not limited
to, the E1545-G1546, E1714-G1715, E1819-A1820, or E1919-L1920
bond (numbering based on the human aggrecan core protein
sequence). These human aggrecan ADMP-sensitive cleavage
10 sites are conserved in aggrecan from various animal
species although the absolute numbering based on the
sequence of the aggrecan core protein may vary from
species to species. Conserved amino acid sequences in
various species around conserved ADMP-sensitive sites are
15 shown below.

	Human	NITEGE ³⁷³	³⁷⁴ ARGSVILT
	Bovine	NITEGE	ARGSVILT
	Rat	NITEGE	ARGNVILT
20	Mouse	NVTEGE	ALGSVILT
	Pig	NITEGE	ARGTVILT
	Sheep	NITEGE	ARGNVILT
	Chicken	NVTEEE	ARGSI
	Horse	NITEGE	ARGNVILT
25			
	Human	ASTASELE ¹⁵⁴⁵	¹⁵⁴⁶ GRGTIGIS
	Bovine	ATTAGELE	GRGTIDIS
	Mouse	ATTSSELE	GRGTIGIS
30	Rat	ATTASELE	GRGTISVS
	Human	PTTFKEEE ¹⁷¹⁴	¹⁷¹⁵ GLGSVELS
	Bovine	PTTFKEEE	GLGSVELS
	Rat	PTTFREEE	GLGSVELS
35	Mouse	PTTFREEE	GLGSVELS
	Human	TQAPTAQE ¹⁸¹⁹	¹⁸²⁰ AGEGPSGI
	Bovine	TQAPTAQE	AGEGPSGI
	Rat	TLAPTAQE	AGEGPSSI
40	Mouse	TQAPTAQE	AGEGPSGI
	Chicken	TQTSVAQE	VGEGPSGM
45	Human	TEPTISQE ¹⁹¹⁹	¹⁹²⁰ LGQRPPVT
	Bovine	TEPTVSQE	LGQRPPVT
	Rat	TEPTVSQE	LGHGPSMT
	Mouse	TEPTVSQE	LGHGPSMT
	Chicken	TRPTVSQE	LGGETAVT
	Dog	TEPTVSQE	LAQRPPVT

5

Thus, aggrecan from various animal species, including but not limited to, bovine, dog, pig, rat, mouse, sheep, horse and chicken may also be used as a substrate for detecting ADMP activity.

The direct ELISA assay employs 96-well filtration plates containing polyvinyl-denedifluoride (PVDF) cationically charged membranes. These membranes are semi-selective in binding the highly negatively-charged aggrecan, which allows for binding of detectable levels of neopeptide antibody-reactive aggrecan fragments from solutions containing high levels of other proteins.

Utilizing neopeptide antibodies allows detection of fragments formed specifically by ADMP-mediated cleavage even in the presence of other proteolytic activities which may be present in crude preparations. Thus, the Problot assay can be used to monitor ADMP activity in culture medium containing other proteases, as well as to monitor the activity of the purified ADMP enzyme.

Therefore, this assay has particular use in following ADMP activity during purification from tissue or media samples as well as for use in enzymatic assays to evaluate inhibitors of the ADMP enzyme. The Problot assay can also be used to detect ADMP-generated aggrecan fragments in culture media from tissue or cell cultures stimulated to induce ADMP-mediated degradation. This assay may also be useful for detecting ADMP-generated aggrecan fragments in cartilage, synovial fluid, serum, urine or other biological samples from patients with ADMP-associated diseases.

Peptide substrates are commonly employed in a variety of assays to determine the presence of enzymes that catalyze the hydrolysis of proteins. One skilled in the art would rely on the use of peptide substrates that are relatively short in length, generally consisting of

5 approximately six to ten amino acids in length. These peptide substrates typically encompass amino acid sequences that bracket the known hydrolysis site of the natural protein substrates. These peptide substrates, including those for matrix metalloproteases, serine
10 proteases, aspartyl proteases, and aminopeptidases, are readily available for use in a variety of enzymatic assays.

This invention provides a peptide that has been found to act as a substrate for the family of ADMPs. It
15 is commonly known that short peptide sequences which contain the proper substrate cleavage site are quite acceptable substrates for many proteases (Copeland, R.A., Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis, VCH/Wiley, New York, 1996).
20 However, no such peptide, even those containing as many as twenty amino acids, has been determined that will act as a suitable substrate for ADMPs. The peptides of the instant invention are unique in that it was unexpectedly found that these longer, forty amino acid sequence acted
25 as very good substrates for ADMPs. One such peptide provided by the invention, of the sequence

QTVTWPDMELPLPRNITEGE-ARGSVILTVKPIFEVSPSPL (SEQ ID NO:1)

30 comprises a 40 amino acid segment of the human aggrecan protein that contains the ITEGE373-374ARGS cleavage site present in the natural protein substrate, aggrecan, and is capable of being cleaved at this specific recognition site by the ADMPs. **Since the human aggrecan ADMP-**
35 **sensitive cleavage sites are conserved in aggrecan from various animal species, peptides based on the amino acid sequence around the ADMP-sensitive cleavage sites from other species can also serve as substrates for ADMPs. A peptide substrate, similar to SEQ ID NO:1, based on a 41**

- 5 amino acid segment of the bovine aggrecan protein, of the sequence

QTVTWPDVELPLPRNITEGE-ARGSVILTAKPDFEVSPTAPE (SEQ ID NO:2)

- 10 containing the E373-A374 cleavage site is also capable of being cleaved at this specific recognition site by the ADMPs.

- Cleavage products are easily detected by using neoeptide antibodies to the N-terminal or C-terminal
15 fragments produced by specific cleavage at the E373-A374 bond, encompassing, but not limited to, the monoclonal antibody BC-3 (Hughes, C.E., et al., Biochem. J. 306:799-804, 1995). The BC-3 antibody recognizes the new N-terminus, ARGS, which is the amino terminal portion of
20 one of the product peptides resulting from the ADMP activity of the enzyme.

- One skilled in the art could readily design peptides of similar size encompassing the alternative ADMP-sensitive cleavage sites in the aggrecan core protein,
25 encompassing, but not limited to, regions of the molecule containing the E1545-G1546, E1714-G1715, E1819-A1820, or E1919-L1920 bond (numbering based on the human aggrecan core protein sequence). One such peptide provided by the invention, of the sequence

- 30 ITFVDTSLVEVTPTTFKEEE-GLGSVELSGLPSGELGVSGT (SEQ ID NO:3)

- comprises a 40 amino acid segment of the human aggrecan protein that contains the KEEE1714-1715GLGS cleavage site
35 present in the natural protein substrate, aggrecan, and is capable of being cleaved at this specific recognition site by the ADMPs.

5 When a preferred form of a peptide substrate, biotinylated at the carboxy terminus **or amino terminus**, is employed, several streptavidin coated supports may be used. These include, but are not limited to microplates, metallic and non-metallic beads, and membranes.

10 Another preferred assay format involves the direct analysis, by high-performance liquid chromatography (HPLC), of the cleavage fragments from the substrate that are generated by ADMP activity.

Another preferred embodiment of the invention
15 provides that a peptide substrate of this invention may be reversed in its role. With proper modification at the P1 position the substrate may be turned into an inhibitor of ADMP activity. Specifically it was found that esterification of the P1 glutamic acid residue (GLU³⁷³) of
20 the substrate peptide SEQ ID NO:1 or its replacement by glutamine abolish catalytic hydrolysis. Unexpectedly, the peptide containing the GLU to GLN substitution at amino acid position 373 (the P1-glutamine containing peptide) was shown to be a competitive inhibitor of the
25 enzyme. Thus, a carboxylate residue at position P1 of the substrate appears to be critical for turnover by ADMPs, but exerts less influence over initial substrate binding to the enzyme. This feature can be readily exploited by one trained in the art to design specific
30 peptide and non-peptide inhibitors of this enzyme.

DEFINITIONS

As used herein, the following terms and expressions have the indicated meanings.

35 The term "aggrecan degrading metallo protease" ("ADMP") activity as referred to herein, refers to the enzymatic activity of a family of polypeptides which specifically cleave the protein aggrecan within the interglobular domain at the Glu³⁷³-Ala³⁷⁴ peptide bond,

5 but do not readily cleave at the Asn341-Phe342 bond which is preferentially cleaved by matrix metalloproteinases.

The term "amino acid" as used herein means an organic compound containing both a basic amino group and an acidic carboxyl group.

10 The term "amino acid residue" as used herein means that portion of an amino acid (as defined herein) that is present in a peptide.

The term "peptide" as used herein means a compound that consists of two or more amino acids (as defined
15 herein) that are linked by means of a peptide bond. The term "peptide" also includes compounds containing both peptide and non-peptide components, such as pseudopeptide or peptide mimetic residues or other non-amino acid components. Such a compound containing both peptide and
20 non-peptide components may also be referred to as a "peptide analog".

The term "peptide bond" means a covalent amide linkage formed by loss of a molecule of water between the carboxyl group of one amino acid and the amino group of a
25 second amino acid.

The term "substrate" refers to a molecule that is bound by the active site and acted upon by the enzyme.

The term "solid-phase peptide synthesis" refers to the direct chemical synthesis of peptides utilizing an
30 insoluble polymeric support as the anchor for the growing peptide, which is built up one amino acid at a time using a standard set of reactions in a repeating cycle (Merrifield, R.B., Science 232, 341-347 1986).

As used herein, the term "TMB" refers to 3,3',5,5'-
35 tetramethylbenzidine.

The term "neoepitope antibody" refers to an antibody which specifically recognizes a new N-terminal amino acid sequence or new C-terminal amino acid sequence generated by proteolytic cleavage but does not recognize these same

5 sequences of amino acids when they are present within the intact protein.

As used herein, the cleavage site "E373-374A" refers to the ITEGE373-374ARGS bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in
10 aggrecan from various animal species, the cleavage site "E1545-1546G" refers to the SELE1545-1546GRGT bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1714-1715G" refers to the
15 KEEE1714-1715GLGS bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1819-1820A" refers to the TAQE1819-1820AGEG bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species, the cleavage site "E1919-1920L" refers to the
20 ISQE1919-1920LGQR bond of human aggrecan as well as to the homologous aggrecanase-sensitive cleavage site in aggrecan from various animal species.

25 The term "aggrecan" as used herein refers to the aggregating proteoglycan, aggrecan, of cartilage from human or various animal species, as the native aggrecan isolated from tissue, as recombinant full-length aggrecan or as a recombinant protein representing a portion of the
30 aggrecan molecule.

As used herein the term "ADMP-susceptible cleavage site" refers to the E373-374A bond, the E1545-1546G bond, the E1545-1546G bond, the E1819-1820A bond, and the E1919-1920L bond of aggrecan from human and various animal
35 species, and to a peptide bond of a protein containing an amino acid sequence which has a glutamine in the P1 position and shows at least 65% homology with the P1, P2, P3, P1', P2' and P3' amino acids of one or more of the ADMP-sensitive sites in the aggrecan molecule.

5 The term "sissel bond" refers to the peptide bond of
a polypeptide that is to be cleaved by a protease. The
term "P1" as used herein refers to the amino acid residue
on the N-terminal side of the sissel bond. The term "P2"
10 as used herein refers to the amino acid residue adjacent
to P1 on the N-terminal side of the sissel bond. The term
"P3" as used herein refers to the amino acid residue
adjacent to P2 on the N-terminal side of the sissel bond.
The term "P1'" as used herein refers to the amino acid
15 residue on the C-terminal side of the sissel bond. The
term "P2'" as used herein refers to the amino acid
residue adjacent to P1' on the C-terminal side of the
sissel bond. The term "P3'" as used herein refers to the
amino acid residue adjacent to P2' on the C-terminal side
of the sissel bond.

20 The term "BC-3 antibody" refers to a monoclonal
antibody that reacts specifically with the newly-formed
amino-terminal sequence ARGS on fragments produced by
proteolytic cleavage at the Glu³⁷³-Ala³⁷⁴ aggrecan
cleavage site, but does not recognize this same sequence
25 of amino acids when they are present within the intact
interglobular domain of the protein (Hughes, C.E., et
al., Biochem. J. 306:799-804, 1995).

 The term "SEQ ID NO:1" refers to the peptide
sequence QVTWPDMEPLPRNITEGE-ARGSVILTVKPIFEVSPSPL. The
30 term "SEQ ID NO:2" refers to the peptide sequence
QVTWPDVELPLPRNITEGE-ARGSVILTAKPDFEVSPTAPE. The term "SEQ
ID NO:3" refers to the peptide sequence
ITFVDTSLEVTPTTFKEEE-GLGSVELSGLPSGELGVSGT. The term "41-
PS" and "SEQ ID NO:4" refer to the peptide sequence:
35 QVTWPDMEPLPRNITEGEARGSVILTVKPIFEVSPSPL-(BIOTINYL)K.
The term "SEQ ID NO:5" refers to the peptide sequence:
ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K. The term "SEQ ID NO:6"
refers to the peptide sequence: K(BIOTINYL)-
QVTWPDMEPLPRNITEGE. The term "30-IP" and "SEQ ID NO:7"

5 refer to the peptide sequence
QTVTWPDMEPLPRNITEGQARGSVILTV-(BIOTINYL)K.

The invention can be further understood by the following examples. These examples provide an illustration of embodiments of the invention and should
10 not be construed to limit the scope of the invention which is set forth in the appended claims. In the following examples all methods described are conventional unless otherwise specified.

15 Example 1

Microplate Assay Format for Detection of ADMP Activity

The substrate and product peptides were prepared in the following manner. A 41 amino acid form (41-PS) SEQ
20 ID NO:4 of the peptide substrate SEQ ID NO:1 was prepared by solid phase peptide synthesis. The peptide was prepared commercially (Quality Controlled Biochemicals, Inc. Hopkinton, MA) as a biotin conjugate by adding an additional lysine residue at the carboxy terminus of the
25 peptide SEQ ID NO:1. Biotin was then covalently attached through the lysine ϵ -amino side chain.

41-PS sequence:

QTVTWPDMEPLPRNITEGE-ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K
30 (SEQ ID NO:4)

A 21 amino acid peptide representing the product of ADMP-mediated cleavage of the 41-PS containing the ARGS N-terminus was prepared in a similar manner and had the following sequence:

35 ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K
(SEQ ID NO:5)

The substrate and product peptide microplates were prepared in the following manner. A 0.1 mM stock of 41-PS was made by dissolving it in distilled water. From
40 this a working solution of 7×10^{-8} M 41-PS was prepared in

5 1X PBS, 0.05% Tween 20. Aliquots of 100 μ L of this solution were added to the microplate wells of a streptavidin coated microtiter strip plate (DUPONT, NEN Products, Catalog # NEF-711). The solutions were allowed to sit at 25°C overnight in order for the biotin moiety of
10 the peptides to bind to the streptavidin which was coated on the microplate. The plate was washed three times with 200 μ L of 1X PBS, 0.05% Tween 20, after which the plate was inverted, blotted dry, sealed and stored at 4°C.

The assay was performed in the following manner.

15 Microplate strips (eight wells each) were rinsed once with 100 μ L of 1X Assay Buffer (Assay Buffer consists of: 50 mM Tris, pH 7.5, 10 mM CaCl_2 , and 100 mM NaCl) and blotted dry. Reactions were prepared in duplicate in a final volume of 100 μ L, containing: 50 μ L of 2X Assay
20 Buffer (100 mM Tris, pH 7.5, 20 mM CaCl_2 , and 200 mM NaCl), 25 μ L of a hydroxymate inhibitor compound (final concentrations consisting of 5.0, 1.0, 0.75, 0.5, 0.25, 0.125, 0.05, 0.001, and 0.0 μ M), and 25 μ L of soluble ADMP (0.05 η M). The microplate strips were incubated for
25 3 hours at 37°C. The microplate wells were then washed six times with 200 μ L of 1X PBS, 0.05% Tween 20 using a Denley Well Wash 4 micro plate washer.

A BC-3 antibody solution was prepared by adding 4 μ L of BC-3 antibody (0.405 mg/mL in PBS) to 2 mL of antibody
30 dilution buffer (DB), which consisted of: 0.1 g BSA (Boehringer Mannheim Catalog 238-031), 10 mL 1X PBS, and 10 μ L Tween 20. 100 μ L of this solution was added to each well. The microplate strips were incubated for 1 hour at 25°C. The microplate wells were washed six times
35 with 200 μ L of 1X PBS, 0.05% Tween 20.

The secondary (detection) antibody solution was prepared by adding 4 μ L of Goat anti-Mouse-HRP antibody conjugate (Pierce Cat# 31430) (0.8 mg/mL in PBS) to 2 mL of antibody dilution buffer (DB). 100 μ L of this

5 solution was added to each well. The microplate strips
were incubated for 1 hour at 25°C. The microplate wells
were washed six times with 200 µL of 1X PBS, 0.05% Tween
20. TMB Substrate(100 µL per well, DAKO Cat S1600) was
added and the microplate strips incubated at 25°C for 15
10 minutes. The reaction was quenched with 100 µL of 1N
HCl. The optical density was read at 450nm using a
Molecular Devices Spectromax 250 microplate reader.
By coating the jplate with the product peptide, one can
create a standard curve which can be used to convert
15 optical density values to units of activity.

ADMP activity can easily be followed by this method
and inhibition of ADMP activity can be monitored. The
IC₅₀ for the inhibition of ADMP by the hydroxamate
inhibitor tested was 0.413 µM.

20

Example 2

HPLC Assay for Detection of ADMP Activity

The High Performance Liquid Chromatography (HPLC)
25 instrument used for the assay was from Hewlett-Packard,
model number HP1090, equipped with a HP ChemStation. A
250 x 4.6 mm Vydac C₁₈ column with 10 µ particle size was
obtained from The Separations Group, Tesperia, CA; HEPES
from Research Organics, Cleveland, OH; Brij-35 from
30 Technicon Corp., Tarrington, NY; Other chemicals from
Sigma, St. Louis, MO; the lyophilized peptide substrate
41-PS, QTVTWPDMEPLRNITEGEARGSVILTVKPIFEVSPSPL-
(BIOTINYL)K (SEQ ID NO:4), which includes the ADMP E373-
A374 cleavage site, was obtained from Quality Controlled
35 Biochemicals, Inc. Hopkinton, MA. A 21 amino acid
product peptide (SEQ ID NO:5) with the sequence,
ARGSVILTVKPIFEVSPSPL-(BIOTINYL)K, used as a standard for
quantitation of product formation, was obtained from the
same vendor.

5 The HPLC assay is performed in the following manner.
The reaction buffer contains 50 mM HEPES, 10 mM CaCl₂,
100 mM NaCl and 0.05% Brij-35, pH 7.5. 30 µM 41-PS was
incubated with 1 unit ADMP activity (1 unit = the amount
of ADMP activity resulting in 1 pMole of 21-mer peptide
10 product produced per hr at 37°) at 37°C for 2.5 hours and
then the reaction was quenched with 50 mM EDTA. A 10 µL
portion of the reaction mixture was injected onto a
reverse-phase HPLC C₁₈ column. The peptides were eluted
with a mobile phase of 0.1% trifluoroacetic acid and a
15 25-45% acetonitrile gradient in 20 minutes. UV
absorbance was measured at 220nm and peak integration was
performed on a Hewlett-Packard HP ChemStation. The 21-
mer product peptide was used as a standard for
quantitation of product formation. The 41-PS and 21-mer
20 product are well separated with retention times of 14.2
and 10.5 minutes, respectively. A standard curve was
prepared using the 21-mer peptide to allow quantitation
of product formation. Effect of incubation time was
evaluated and found to be linear over the timecourse of
25 the assay (Figure 2).

Example 3

Inhibition of ADMP Activity by a Peptide Inhibitor

30 A peptide inhibitor was prepared based upon the
sequence of the 40 amino acid peptide SEQ ID NO:1, but
designed such that it contained a Glu to Gln substitution
at the P1 of the Glu373-Ala374 bond. This peptide (30-
IP) of the sequence:

35 QTVTWPDMELPLPRNITEGQARGSVILTVK-Biotin

(SEQ ID NO:7)

was prepared with the n-terminus acetylated and the c-
terminal residue present as the amide (Quality Controlled
Biochemicals, Inc., Hopkinton, MA). This peptide was

5 employed in a microplate assay as described in Example 1 wherein the 30-IP inhibitor was substituted for the hydroxamate inhibitor used in that example. The 30-IP inhibitor was employed at final concentrations of 0.01, 0.1, 1.0, 3.0, 5.0, 10.0, 30.0 and 100.0 μ M.

10 ADMP activity was inhibited as shown in Figure 3. The IC₅₀ for the inhibition of ADMP activity by 30-IP was 11 μ M.

EXAMPLE 4

15 Problot Assay

This example describes a method for analyzing ADMP enzymatic activity and inhibitors of this activity by monitoring cleavage at the E373-A374 bond using the BC-3
20 antibody to detect fragments with the new N-terminus, ARGS. Samples containing ADMP activity (10 units/ml) were incubated with 500 nM bovine aggrecan monomer in a final volume of 200 μ L in 0.05 M Tris, pH 7.6, containing 0.1 M NaCl and 10 mM CaCl₂. Reactions were incubated for
25 4 hr at 37°C, quenched with 20 mM EDTA, and analyzed for aggrecan fragments with the new N-terminus, ARGS, generated by specific ADMP-mediated cleavage using the Problot assay.

The Immobolin PVDF membrane plate (#MAIPN4550;
30 Millipore Corp., Bedford, MA) was prewet with 50 μ L per well 70% ethanol, incubated for 30 seconds at room temperature then flushed two times each with 200 μ L of purified H₂O. The plate was then coated with aggrecan equivalent to 36 μ g of glycosaminoglycan (GAG) as
35 detected by the dimethyl methylene blue dye assay [Farndale R.W., et al., (1982) Conn. Tiss. Res. 9, 247-248.] in 150 μ L of 50 mM carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C with gentle agitation. The coating solution was then filtered off of the plate using a
40 vacuum manifold and membranes were washed once with 200 μ L of Buffer A (Buffer A comprises 20 mM Tris, 500 mM

5 NaCl, pH 7.5) allowing 20 seconds of contact with the
membrane. Membranes were then blocked with 150 μ L of 5%
BSA/TBS solution for 1 hour at room temperature with
gentle agitation. The blocking solution was filtered off
of the plate and the membranes washed one time with 200
10 μ L of 1X TBS buffer, allowing 20 seconds of contact with
membrane per wash.

Removal of the glycosaminoglycan (GAG) side chains
from aggrecan is necessary for the BC-3 antibody to
recognize the epitope on the core protein. Therefore, to
15 remove GAGs from the bound aggrecan, samples were treated
with deglycosylation enzymes as follows: 0.01 units
chondroitinase ABC (#EC4.2.2.4; Seikagaku Co., Kogyo,
Japan) per 10 μ g GAG in 150 μ L of Buffer B (Buffer B
comprises 50 mM sodium acetate, 100 mM NaCl, pH 6.5) was
20 added to each well and incubated at 37°C for 1 hour.
Following incubation the enzyme solution was filtered out
of the plate and 0.01 units chondroitinase ABC per 10 μ g
GAG, 0.01 units keratanase I (#EC3.2.1.103; Co., Kogyo,
Japan) per 10 μ g GAG, and 0.0005 units keratanase II
25 (Seikagaku Co., Kogyo, Japan) per 10 μ g GAG in 150 μ L
Buffer B were added and allowed to incubate an additional
2 hours at 37°C. Enzyme solution was filtered out and
membranes rinsed one time with 200 μ L of Buffer A.

150 μ L of BC-3 antibody was added at a 1:500
30 dilution in 1% BSA in Buffer A and incubated for 1 hour
at room temperature with gentle agitation. BC-3 antibody
was removed and membranes washed three times each with
200 μ L Buffer A allowing membrane contact for 20 seconds
per wash. Next 150 μ L of goat anti-mouse IgG AP
35 conjugate (#S3721; Promega, Madison, WI) was added at a
1:2500 dilution in 1% BSA/TBS buffer and allowed to
incubate for 1 hour at room temperature with gentle
agitation. The secondary antibody was prefiltered prior
to use, using 0.22 μ M syringe filters to remove
40 aggregates which cause high background. Following the
incubation, secondary antibody was removed and wells were
washed three times each with 200 μ L Buffer A allowing

5 contact for 20 seconds per wash. Then 100 μ L of p-NPP AP
substrate solution (# 50-80-00; Kirkegaard & Perry Lab.,
Gaithersburg, MD) was added to each well and incubated in
the dark at room temperature for 30 minutes. The
solution was then filtered into a corresponding ELISA
10 plate. The filter plate was washed with 100 μ L of 500 mM
EDTA and the wash was combined with the corresponding
samples in the ELISA plate. Absorbance of the samples
was read at 405 nm (Thermomax plate reader).

By coating the plate with the target peptide, ARGS,
15 linked to BSA representing the ADMP-generated product,
one can create a standard curve which can be used to
convert optical density values to units of activity. A
unit of ADMP activity is defined as that resulting in
product produced equivalent to 1 μ g BSA peptide per hour
20 at 37°C.

To evaluate inhibition of ADMP activity, compounds
are prepared as 10 mM stocks in dimethyl sulfoxide
(DMSO), water or other solvents and diluted to
appropriate concentrations in water. Drug (50 μ L) was
25 added to 50 μ L of 2 mg/mL aggrecan substrate and 50 μ L of
ADMP (40 units/ml) and brought to a final volume of 200
 μ L by addition of 50 μ L of 0.2 M Tris, pH 7.6,
containing 0.4 M NaCl and 40 mM CaCl_2 . The reaction
mixture was incubated for 4 hr at 37°C, quenched with 20
30 mM EDTA and analyzed for ADMP-generated products. A
sample containing enzyme and substrate without drug was
included as a positive control and enzyme prequenched
with EDTA served as a measure of background.

IC₅₀ values for inhibitors of ADMP enzymatic
35 activity determined using the Problot assay with the BC-3
antibody for analysis of product generation by cleavage
at the E373-A374 bond correlated ($r^2=0.99$) with those
determined using a BC-3 Western blot analysis to detect
product formation.

40

5

CLAIMS

What is claimed is:

1. A peptide containing a specific ADMP-susceptible
cleavage site.
- 10 2. A peptide of claim 1 wherein the peptide has a
linking-moiety.
3. A peptide comprising a sequence of
15 amino acids 1-40 of SEQ ID NO:1.
4. A peptide comprising a sequence of amino acids that
is at least 80% identical to the sequence of amino
acids 1-40 of SEQ ID NO:1.
- 20 5. A peptide of comprising a sequence of
amino acids 1-40 of SEQ ID NO:2.
6. (13) A peptide comprising a sequence of
25 amino acids 1-40 of SEQ ID NO:3.
7. (14) A peptide comprising a sequence that
is at least 80% identical to the sequence of amino
acids 1-40 of SEQ ID NO:3.
- 30 8. A peptide of claims 1, 2, 3, 4, 5, 6 or 7 wherein the
peptide is biotinylated.
9. A peptide of claim 2 wherein the linking-moiety is a
35 biotinylated lysine.
10. A peptide of claim 2 wherein the linking-moiety
contains a chromophore.

- 5 11. A peptide of claim 2 wherein the peptide
has a C-terminal linking-moiety.
12. A peptide of claim 2 wherein the peptide has a C-
terminal linking-moiety that is a biotinylated
10 lysine.
13. A peptide of claim 2 wherein the peptide
has an N-terminal linking-moiety.
- 15 14. A peptide of claim 2 wherein the peptide has an N-
terminal linking-moiety that is a biotinylated
lysine.
15. A product peptide of claim 1 comprising the amino
20 acids from the N-terminus through P1 of the ADMP-
susceptible cleavage bond.
16. A product peptide of claim 1 comprising the amino
acids from P1' of the ADMP-susceptible cleavage bond
25 through C-terminus.
17. A peptide of claims 15 or 16 wherein the peptide
is biotinylated.
- 30 18. A peptide of claim 15 wherein the peptide has an N-
terminal linking-moiety.
19. A peptide of claim 16 wherein the peptide has a C-
terminal linking-moiety.
35
20. A peptide of claim 18 wherein the linking-
moiety is a biotinylated lysine.

- 5 21. A peptide of claim 19 wherein the linking-
moiety is a biotinylated lysine.
22. A peptide of claim 18 wherein the linking-
moiety contains a chromophore.
- 10 23. A peptide of claim 19 wherein the linking-
moiety contains a chromophore.
- 15 24. A peptide comprising a sequence of amino acids 20-40
of claim 3, wherein an additional biotinylated
lysine is attached to the C-terminus via a peptide
bond, comprising a sequence of amino acids of SEQ ID
NO:5.
- 20 25. A peptide comprising a sequence of amino acids 1-20
of claim 3, wherein an additional biotinylated
lysine is attached to the N-terminus via a peptide
bond, comprising a sequence of amino acids of SEQ ID
NO:6.
- 25 26. A method for the determination of the presence of
aggrecan-degrading metalloprotease activity
comprising:
- 30 (a) binding an ADMP substrate peptide of claim
1 to a streptavidin-coated microtiter
plate;
- (b) rinsing the microtiter plate with assay
buffer;
- 35 (c) incubating the microtiter plate with an
ADMP-containing sample;
- (d) rinsing the microtiter plate;
- (e) incubating the microtiter plate with a
neoepitope antibody solution;
- (f) rinsing the microtiter plate;

- 5 (g) incubating microtiter plates with
 secondary-detection antibody solution;
 (h) incubating the microtiter plate with an
 appropriate substrate solution;
 (i) quenching the reaction;
10 (j) reading the optical density;

27. The method of claim 26, wherein said ADMP peptide
 substrate comprises a covalently-linked linking-
 moiety.

15

28. A method for the determination of ADMP activity by
 quantifying the appearance of a product peptide
 comprising:

- 20 (a) incubating an ADMP substrate peptide of
 claim 1 with assay buffer and ADMP-
 containing sample;
 (b) quenching the reaction;
 (c) injecting a portion of the reaction mixture
 onto a reverse-phase HPLC column;
25 (d) eluting the peptide with an organic
 solvent;
 (e) reading the absorbance;
 (f) determining the quantity based on a
 standard curve.

30

29. A method for assaying compounds for activity against
 an ADMP comprising:

- 35 (a) providing an ADMP and an ADMP substrate;
 (b) contacting said ADMP with a candidate
 inhibitory compound in the presence of
 said ADMP; and
 (c) measuring the inhibition of the ADMP
 activity.

- 5 30. A method for assaying compounds according
to claim 29 wherein the ADMP activity is monitored
according to claim 26 or 28.
- 10 31. A peptide of claim 3, 4, or 5 wherein the P1 amino
acid residue, Glu, the ADMP-sensitive Glu373-Ala374
bond, is esterified.
- 15 32. A peptide of claim 3, 4, or 5 wherein the P1 amino
acid residue, Glu, of the ADMP-sensitive Glu373-
Ala374 bond, is replaced with a Gln amino acid
residue.
- 20 33. An assay for detecting ADMP activity which comprises:
(a) incubating a sample containing
soluble ADMPs or aggrecanase activity with
an aggrecan substrate; and
(b) monitoring production of
aggrecan fragments produced by specific
25 cleavage at an ADMP-susceptible site using
a neoepitope antibody to the new N-
terminus or the new C-terminus generated
by specific ADMP-mediated cleavage by the
Problot assay comprising:
(1) incubate a
30 polyvinyl-
denedifluoride (PVDF) cationically
charged membrane, secured in a welled
filtration plate, with a sample
containing ADMP-degraded aggrecan;
35 (2) wash any unbound aggrecan from
the filtration plate;
(3) couple any unreacted cationic
sites on the PVDF membrane with a

5 solution of bovine serum albumin
(BSA);
(4) wash any unbound BSA from the
filtration plate;
(5) remove glycosaminoglycan side
10 chains from the bound aggrecan with
deglycosylation enzymes, wash
membrane;
(6) incubate PVDF membrane with a
neoepitope antibody to fragments
15 generated by cleavage at an ADMP-
sensitive site, wash membrane;
(7) incubate PVDF membrane with
secondary detection antibody, wash
membrane;
20 (8) incubate PVDF membrane with
detection substrate;
(9) drain solution into wellled plate,
obtain absorbance readings on
individual samples; compare values to
25 those obtained for standard curve.

34. A method for assaying compounds according to claim 29
wherein ADMP activity is monitored according to
claim 33.
- 30 35. An assay according to claim 33 wherein the tissue
or cell source of ADMPs is cartilage or
chondrocytes.
- 35 36. An assay according to claim 33 or 34 wherein the
aggrecan substrate is native aggrecan isolated from
human or animal tissue.

- 5 37. An assay according to claim 33 or 34 wherein the
aggrecan substrate is a recombinant aggrecan
molecule or recombinant portion of the aggrecan
molecule containing an aggrecanase-sensitive
cleavage site.
- 10 38. An assay according to claim 33 or 34 wherein the
recombinant portion of the aggrecan molecule
contains the E³⁷³⁻³⁷⁴A bond.
- 15 39. An assay according to claim 33 or 34 wherein the
recombinant aggrecan fragment contains the E¹⁵⁴⁵⁻¹⁵⁴⁶G
bond.
- 20 40. An assay according to claim 33 or 34 wherein the
portion of the aggrecan molecule contains the E¹⁷¹⁴⁻¹⁷¹⁵G bond.
- 25 41. An assay according to claim 33 or 34 wherein the
recombinant portion of the aggrecan molecule
contains the E¹⁸¹⁹⁻¹⁸²⁰A bond.
- 30 42. An assay according to claim 33 or 34 wherein the
recombinant portion of the aggrecan molecule
contains the E¹⁹¹⁹⁻¹⁹²⁰L bond.
- 35 43. A method according to claims 26, 30, 33, or 34
wherein the neoepitope antibody recognizes the new
N-terminus or new C-terminus generated by cleavage
at the E³⁷³⁻³⁷⁴A bond.
44. A method of any of claims 26, 30, 33, or 34 wherein
the neoepitope antibody is the BC-3 monoclonal
antibody.

- 5 45. A method of any of claims 26, 30, 33, or 34
 wherein the neoepitope antibody recognizes the new
 N-terminus or new C-terminus generated by cleavage
 at the E1545-G1546 bond.
- 10 46. A method of any of claims 26, 30, 33, or 34
 wherein the neoepitope antibody recognizes the new
 N-terminus or new C-terminus generated by cleavage
 at the E1714-G1715 bond.
- 15 47. A method of any of claims 26, 30, 33, or 34 wherein
 the neoepitope antibody recognizes the new N-
 terminus or new C-terminus generated by cleavage at
 the E1819-A1820 bond.
- 20 48. A method of any of claims 26, 30, 33, or 34 wherein
 the neoepitope antibody recognizes the new N-
 terminus or new C-terminus generated by cleavage at
 the E1919-L1920 bond.
- 25 49. A method of use of the assay in claim 33 for
 detecting ADMP-generated aggrecan fragments in
 culture media from tissue or cell cultures
 stimulated to induce aggrecanase-mediated
 degradation.
- 30 50. A method of use of the assay in claim 33 for
 detecting aggrecanase-generated aggrecan fragments
 in biological fluids, tissue extracts or
 homogenates, serum or urine from patients with
35 aggrecanase-associated diseases.
51. A method for diagnosing arthritic diseases in a
 mammal by monitoring ADMP-generated aggrecan
 fragments according to claims 33.

5

52. A method for diagnosing a disease in a mammal
characterized by overproduction or up-regulated
production of an ADMP by monitoring fragments
generated at an ADMP-sensitive site according to
claims 33.

10

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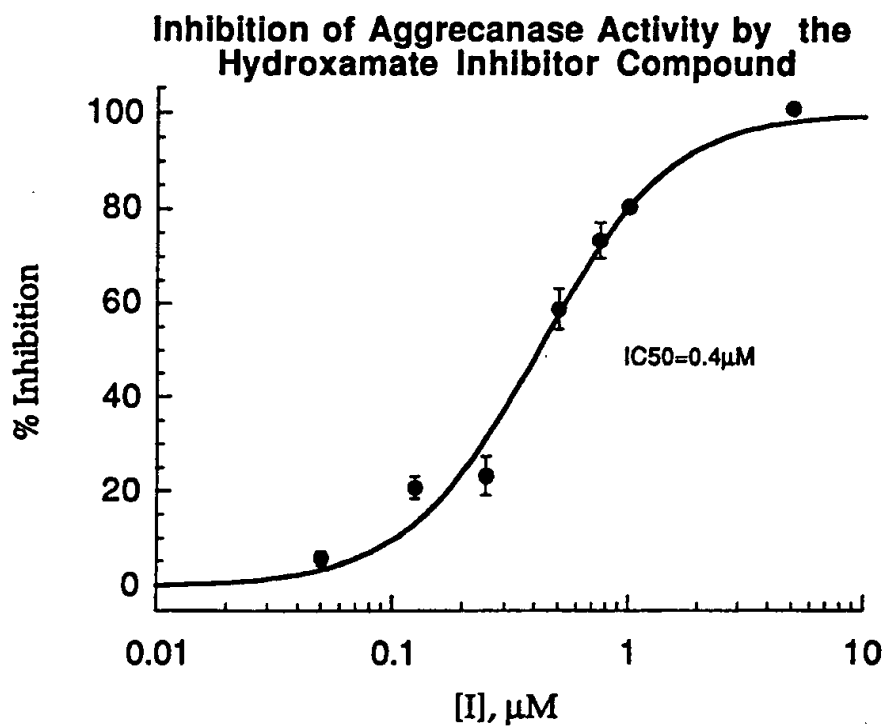
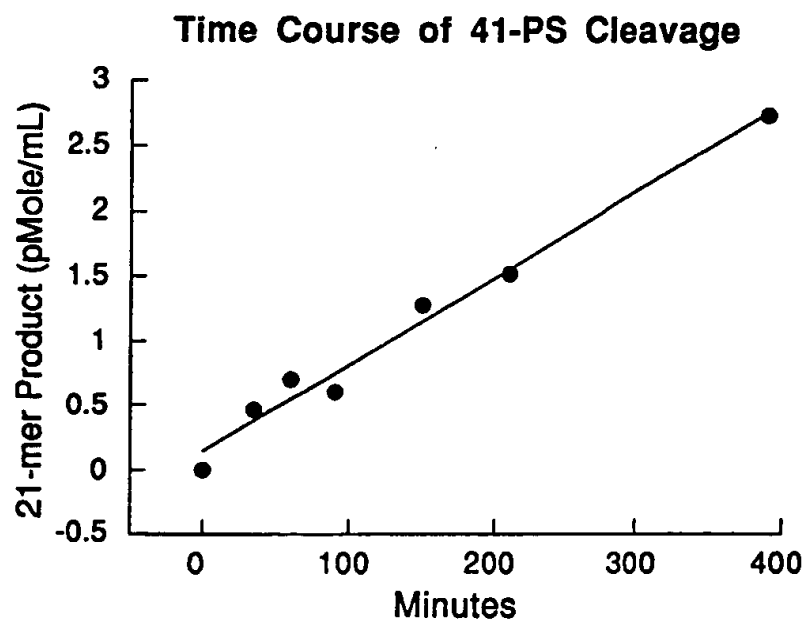
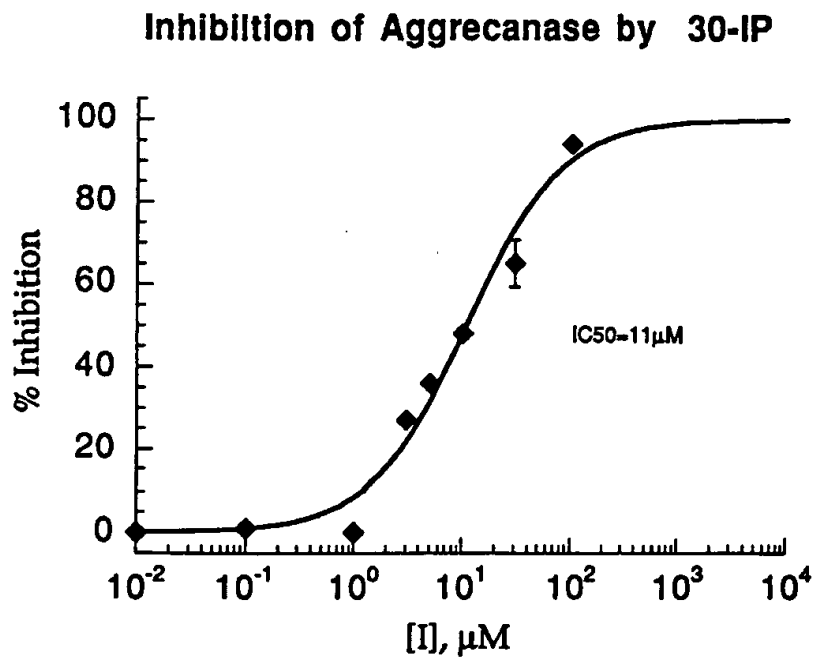


Figure 1

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**Figure 2**

3/3

**Figure 3**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/15436

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C07K14/47 G01N33/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 785 274 A (HOECHST AG) 23 July 1997 see SeqId. No. 3 see page 3, line 7 - line 40; claims ---	1-4, 15, 16, 26, 35
X	EMBL/GENEBANK DATABASES, Access no. P13608, Sequence reference PGCA_BOVIN, 01-january-1990, "Aggreccan core Protein precursor" XP002100469 see the whole document ---	5-7
A	WO 96 01847 A (UNIV MELBOURNE ;FOSANG AMANDA JANE (AU)) 25 January 1996 see claims; examples --- <div style="text-align: center;">-/--</div>	33-52
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">20 Apr11 1999</div>		Date of mailing of the international search report <div style="text-align: center;">06/05/1999</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Fuhr, C</div>

INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/US 98/15436

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 97 25437 A (TNO ;TE KOPPELE JOHANNES MARIA (NL); BEEKMAN BOB (NL)) 17 July 1997 see page 7, line 9 - page 9, line 7; claims -----	1,2,10, 11,18, 22,23

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Information on patent family members

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